PB86-187234

Screening Complex Hazardous Wastes for Mutagenic Activity Using a Modified Version of the TLC/Salmonella Assay

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1986

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA/600/J-86/024	2.	3. PB86-187234
4. TITLE AND SUBTITLE Screening Complex Hazardous Wastes for Mutagenic Activity Using a Modified Version of the TLC/ Salmonella Assay (Journal Version)		5. REPORT DATE 1986 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Virginia Stewart Houk Larry D. Claxton		8. PERFORMING ORGANIZATION REPORT NO
9. PERFORMING ORGANIZATION NAME AND ADDRESS GBB/GTD/HERL MD68 Research Triangle Park, NC 27711		10. PROGRAM ELEMENT NO 11. CONTRACT/GRANT NO
12. SPONSORING AGENCY NAME AND ADDRESS HEALTH EFFECTS RESEARCH LABORATORY OFFICE OF RESEARCH AND DEVELOPMENT U.S. ENVIRONMENTAL PROTECTION AGENCY RESEARCH TRIANGLE PARK, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED Journal Article 14. SPONSORING AGENCY CODE EPA/600/11

15. SUPPLEMENTARY NOTES
Mutation Research, 169 (1986) 81-92 March
Elsevier Science Publishers B. V.

16. ABSTRACT

Ten complex hazardous wastes were tested for mutagenic activity using a modified version of the TLC/Ames assay developed by Bjorseth et al. (1982). This fractionation/bioassay scheme couples thin layer chromatography (TLC) with the Salmonella/mammalian-microsome (Ames) assay for the detection of mutagenic constituents in complex mixtures. Crude (unadulterated) hazardous wastes and selected hazardous waste extracts were fractionated on commercially available cellulose TLC plates. Mutagenicity testing was performed in situ by applying a single overlay of minimal growth agar, tester strain TA98 or TA100, and the optional metabolic activation system directly onto the developed chromatogram. A mutagenic effect was indicated either by the appearance of localized clusters of revertant colonies or by an increase in total revertant growth vis-a-vis control plates. Seven of ten hazardous wastes (including tars, emulsions, sludges, and spent acids and caustics) demonstrated mutagenic activity when tested by this method.

To assess the sensitivity of the modified TLC/Ames assay, fourteen Salmonella mutagens from a wide range of chemical classes and polarities were tested. Chemicals selected included heterocyclics, aromatic amines, alkylating agents, antitumor agents, a nitrosamine, and a nitroaromatic. Eleven of the fourteen control compounds were positive in this test system. The three compounds refractory to analysis included a polycyclic aromatic hydrocarbon and two volatiles.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS (This Report) UNCLASSIFIED	21. NO. OF PAGES 13
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA/600/J-86/024
JOURNAL ARTICLE

Mutation Research, 169 (1986) 81–92
Elsevier

MTR 01041

Screening complex hazardous wastes for mutagenic activity using a modified version of the TLC/Salmonella assay

Virginia Stewart Houk [1,2] and Larry D. Claxton [1]

[1] *Genetic Bioassay Branch, GTD/HERL, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711 and*
[2] *Research performed under MSPH degree program, University of North Carolina School of Public Health, Chapel Hill, NC 27514 (U.S.A.)*

(Received 22 May 1985)
(Revision received 4 September 1985)
(Accepted 23 September 1985)

Summary

10 complex hazardous wastes were tested for mutagenic activity using a modified version of the TLC/Salmonella assay developed by Bjørseth et al. (1982). This fractionation/bioassay scheme couples thin-layer chromatography (TLC) with the Salmonella/mammalian-microsome (Ames) assay for the detection of mutagenic constituents in complex mixtures. Crude (unadulterated) hazardous wastes and selected hazardous waste extracts were fractionated on commercially available cellulose TLC plates. Mutagenicity testing was performed in situ by applying a single overlay of minimal growth agar, tester strain TA98 or TA100, and the optional metabolic activation system directly onto the developed chromatogram. A mutagenic effect was indicated either by the appearance of localized clusters of revertant colonies or by an increase in total revertant growth vis-à-vis control plates. 7 of 10 hazardous wastes (including tars, emulsions, sludges, and spent acids and caustics) demonstrated mutagenic activity when tested by this method.

To assess the sensitivity of the modified TLC/Salmonella assay, 14 Salmonella mutagens from a wide range of chemical classes and polarities were tested. Selected compounds included heterocyclics, aromatic amines, alkylating agents, antitumor agents, a nitrosamine and a nitroaromatic. 11 of the 14 mutagens were positive in this test system. The 3 compounds refractory to analysis included a polycyclic aromatic hydrocarbon and two volatiles.

Complex hazardous wastes are composed of an array of compounds which manifest diverse chemical, physical and toxicological properties. Human exposures to hazardous wastes have been associated with a number of adverse health effects, including chromosomal damage, cancer and reproductive anomalies (Maugh, 1979; Vianna and Polan, 1984; Warren, 1981). Adequate characterization of the genotoxic potential of hazardous wastes is therefore crucial to the assessment of risk to human health.

Assessments of genotoxic risk are based in part upon results from short-term in vitro assays. One such test, the Salmonella/mammalian-microsome assay (Ames et al., 1975) has been used for over a

CAS Registry Numbers: 2-acetylaminofluorene, 53-96-3; 2-anthramine, 610-49-1; benzo[*a*]pyrene, 50-32-8; cyclophosphamide, 50-18-0; daunomycin, 20830-81-3; dimethylcarbamyl chloride, 79-44-7; epichlorohydrin, 106-89-8; methyl methanesulfonate, 66-27-3; *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, 70-25-7; 2-nitrofluorene, 60757-8; β-propiolactone, 57-57-8; sodium azide, 26628-22-8; streptozotocin, 18883-66-4.

0165-1218/86/$03.50 © 1986 Elsevier Science Publishers B.V. (Biomedical Division)

NTIS is authorized to reproduce and sell this report. Permission for further reproduction must be obtained from the copyright owner.

decade to predict the mutagenic potential of pure compounds and complex mixtures. This mutagenicity assay has been applied to a variety of municipal and industrial wastes, including municipal wastewaters and sludges (Clevenger et al., 1983; Hopke et al., 1982; Rappoport et al., 1979), industrial process streams and discharges (Andon et al., in press; Bjørseth et al., 1979; Douglas et al., 1980; Kamra et al., 1983; Nestmann et al., 1980; Rannug et al., 1981; Somani et al., 1980; Stinnett et al., 1981) and effluents from energy-related technologies (Epler et al., 1977, 1978a, b; Rao et al., 1978).

The mutagenic activity of a complex mixture can be masked by toxicity or by compound interactions which interfere with mutagenic expression. To enhance detection capabilities, bioassays are often coupled with chemical or physical fractionation techniques to separate or remove toxic components from the complex sample and to reduce the effects of interactive mechanisms.

One simple, yet sensitive, method of fractionation is thin-layer chromatography (TLC). TLC has been successfully coupled with the Salmonella reverse mutation assay to isolate and identify potential mutagens in a variety of complex environmental mixtures, including airborne pollutants (Commoner et al., 1978; Dehnen et al., 1977; Pederson and Siak, 1980; Pitts et al., 1978), drinking water (Tabor and Loper, 1980), and fossil fuels (Pelroy and Wilson, 1981; Wilson et al., 1980). In each study, chromatographed compounds were eluted from the TLC plates prior to bioassay.

A strategy that eliminates the need for elution of compounds from TLC plates has been proposed by Bjørseth et al. (1982). Their technique (the TLC/Salmonella assay) prescribes direct application of the Salmonella assay onto the developed chromatogram. The subsequent appearance of localized clusters of revertant colonies suggests the presence of a mutagenic constituent. This assay has been successfully applied to the analysis of air samples (Bjørseth et al., 1982), extracts of typewriter ribbons and carbon paper (Bjørseth et al., 1982; Møller et al., 1983), and emission samples from coal- and oil-fired boilers (Alfheim et al., 1983).

We modified the TLC/Salmonella assay described by Bjørseth and his colleagues by replacing the double-agar system with a single overlay composed of agar, tester strain, and the metabolic activation system. This modification simplified the technique and reduced preparation time. In addition, the single overlay provided direct contact between bacteria and partitioned components on the chromatogram, ostensibly enhancing detection of nonpolar and intermediately polar compounds.

Ten hazardous wastes were screened for mutagenic activity using this modified assay. Samples were selected from a variety of dissimilar waste types, including, acids, caustics, tars, emulsions and sludges. Solids, semi-solids and liquids were represented, as were inorganic and organic wastes.

The detection capabilities of this modified TLC/Salmonella assay were challenged with 14 compounds known to be mutagenic in the Salmonella plate-incorporation assay. Mutagens were selected from a wide range of chemical classes and polarities, and included alkylating agents, aromatic amines, antitumor agents, a nitrosamine and a nitroaromatic.

Materials and methods

Waste samples

Table 1 provides gross chemical and physical characterizations and a cursory description of each waste tested. Results from gas chromatography/mass spectrometry (GC/MS), emission spectroscopy, and analysis for purgeable and semi-volatile organic compounds have been published elsewhere (Battelle, 1981; Houk, 1984; Miller et al., 1981; Warner et al., 1981).

The organic sludge sample represents Quality Assurance Sludge Material collected by the National Bureau of Standards of the U.S. Department of Commerce. The sludge was obtained from an industrial solvent recovery process. Its spiked counterpart represents a 2% solution (by weight) of the parent sludge material fortified with 11 organic compounds, including mutagens/carcinogens such as fluoranthene (10.99 μg/g), benzidine (10.69 μg/g), and benz[*a*]anthracene (11.14 μg/g) (U.S. Department of Commerce, 1983). Samples arrived in amber glass bottles with Teflon®-lined caps and were stored at room temperature in an air-tight cabinet.

The remainder of the waste samples were ob-

TABLE 1

DESCRIPTION OF HAZARDOUS WASTE SAMPLES

Waste	Physical state	pH [a]	Total solids [a] (%)	Description
Organic sludge	Liquid	NM	NM	Sludge from an industrial solvent recovery process
Organic sludge, spiked	Liquid	NM	NM	Sludge described above, diluted and fortified with 11 organics
EDC spent caustic (water portion)	Liquid	5.2	<1.0	Clear, tan liquid waste from ethylene dichloride production
Herbicide mfg. acid	Liquid	0.5	42	Thin, gray aqueous suspension from an herbicide production process
Coke plant waste	Liquid	8.0	4	Thin, brown fluid with suspended solids, 95% DMSO-soluble, water-insoluble
Ink pigment sludge	Semi-solid	8.7	14	Pourable black jelly from ink production
Latex paint mfg. waste	Suspension	7.5	41	Thick polymeric emulsion of pigment, 50% soluble in DMSO
Coal gasification tar	Tar	8.1	79	Black, viscous tar produced during coal gasification
Organic still bottoms	Solid	3.8	83	Thick, black slurry from tank bottoms
Dewatered municipal sludge	Solid	7.5	44	Dark, wet cake of chemically treated municipal sludge
Oil-refining waste	Semi-solid	7.0	59	Dark liquid with black flocculent and oil droplets, DMSO- and water-soluble

[a] Analysis performed by Research Triangle Institute (Sparacino, 1983).
NM, not measured.

tained from Battelle Columbus Laboratories courtesy of Dr. M. McKown. Each waste contained a variety of potentially hazardous compounds present in detectable amounts (Battelle, 1981). Prior to arrival at our laboratory, wastes had been stored in the dark at 5°C in amber glass bottles. Upon receipt, samples were stored at room temperature in an air-tight cabinet.

Most samples arrived in physical states that favored direct application to TLC plates (liquids, emulsions, sludges and a tar). The organic still bottoms and the dewatered municipal sludge, however, were solids and could not be applied to plates in their unadulterated states. Ethanol (EtOH) extracts of these wastes were tested. Ethanol and dimethyl sulfoxide (DMSO) extracts of the coke plant waste were also obtained and were tested in addition to the crude sample. Extractions were performed as previously described (Andon et al., in press).

The TLC / Salmonella technique

TLC separations were performed on glass-backed cellulose plates, 10 by 10 cm (Alltech). Plates were sterilized prior to sample application according to the procedure described by Bjørseth et al. (1982).

Waste samples were applied to TLC plates with microliter syringes (Unimetrics Corporation). Samples were spotted at 2-cm intervals across the plates at a distance 1.5 cm from the bottom edge. 4 volumes were applied across the plate, each volume doubling the one preceeding it. Doses ranged from approximately 0.1 to 16 μl per application point.

Plates were lowered vertically into a saturated

developing chamber with the mobile phase solvent at a depth of 1 cm. A mobile phase of intermediate polarity was chosen because samples were of unknown polarity and of varied chemical character. Chloroform was selected for initial chromatographic runs. When chloroform proved ineffective in fractionating the sample, other carrier systems were utilized (see Results and Discussion). Elution capabilities were changed by simply increasing or decreasing the polarity of the mobile phase.

Plates were developed at room temperature and were removed from the developing chamber when the solvent front had ascended to approximately 1 cm from the top edge of the plate (10–20 min). All traces of the solvent were passively evaporated from the plates prior to mutagenesis testing.

Developed plates were inspected for chromatographic patterns using two non-destructive visualization processes. Intrinsically colored zones were identified under ambient lighting. Fluorescent banding patterns were detected by short-wave (254 nm) and long-wave (366 nm) ultraviolet light sources.

Chromatograms were placed sorbent side up into over-sized Petri dishes (150 mm by 15 mm) for mutagenicity testing. Top corners of the chromatograms were excised by a glass cutter for proper fit. Genotoxic analyses were performed in situ.

Salmonella typhimurium strains TA98 and TA100 were obtained from Dr. Bruce Ames. Overnight cultures were grown in nutrient broth (Oxoid) at 37°C. Rat-liver homogenate (S9) was prepared from rats injected with Aroclor 1254 as previously described (Ames et al., 1975). Developed blank TLC plates were used as negative controls. Undeveloped plates spotted with 2-anthramine, 2-nitrofluorene or sodium azide were used as positive controls.

Whereas Bjørseth et al. (1982) tested for mutagenicity with a double-agar system composed of minimal bottom agar and a top agar overlay, we designed a single overlay that combined minimal agar, the tester strain and the metabolic activation system. In theory, the presence of the Salmonella bacteria contiguous to the plate should facilitate the detection of nonpolar and intermediately polar compounds that do not readily diffuse through a bottom agar layer.

Because the single overlay method disperses the tester strain throughout a larger volume of agar than that found in the double-agar system, more bacteria per ml of agar are needed in order to position an equivalent number of bacteria over the TLC plate. Calculations showed that 500 µl of the overnight culture were required (as compared to the 300 µl used in the double-agar approach) to compensate for this dilution. Accordingly, 500 µl of tester strain were added along with 1.5 ml of the optional S9 mix to 30 ml of 1.5% minimal agar containing traces of histidine and biotin (Claxton et al., 1981) at 45°C. Overlay contents were vortexed and carefully poured over the plates in the Petri dishes. Dishes were rotated for even distribution of the overlay. After the agar had solidified, dishes were inverted and incubated at 37°C for 72 h.

Due to the qualitative nature of this screening assay, testing was performed on single plates. Dose–response relationships were examined by applying 4 different volumes per plate. Independent experiments were performed to guarantee reproducibility and to confirm positive or negative findings. Results were documented by tracing observed chromatographic patterns and revertant colony locations onto transparent sheets of cellulose acetate.

To examine the predictive capabilities of the assay, 14 Salmonella mutagens of various classes and polarities were selected for testing. Each compound was diluted to 2 mg/ml with analytical grade methylene chloride. Increasing volumes of the mutagen were spotted in clockwise fashion at the 4 corners of a cellulose plate (Table 3). Plates were not chromatographed, but were placed undeveloped into petri dishes and tested for mutagenic response using the single overlay previously described.

Results and discussion

Analysis of hazardous wastes

It should be noted that wastes tested in this study were selected based on availability of the sample. They were not chosen because of specific health-related effects. Because only one waste was evaluated from each waste category, observed results should not be extrapolated to other wastes of that class. Brief preliminary summaries of some of

TABLE 2

RESULTS FROM THE TLC/SALMONELLA ANALYSIS OF HAZARDOUS WASTES

Waste	Extract [a]	TLC plate dose range (μl/application)	Mutagenicity			
			TA98		TA100	
			+S9	−S9	+S9	−S9
Organic sludge	NEAT	0.25–2	+ [b]	+ [c]	+ [d]	−
Organic sludge, spiked	NEAT	0.25–16	−	+ [d]	−	−
EDC spent caustic	NEAT	0.5–8	−	+ [c]	−	+ [b,c]
Herbicide mfg. acid	NEAT	0.1–4	+ [b,c]	−	+ [b,c]	−
Coke plant waste	NEAT	0.5–10	−	+ [b]	−	−
	EtOH	0.5–6	+ [c]	−	−	−
	DMSO	0.5–8	−	−	−	+ [d]
Ink pigment sludge	NEAT	0.5–4	−	−	incl	incl
Latex paint mfg. waste	NEAT	0.5–12	−	−	−	−
Coal gasification tar	NEAT	unknown	+ [b,c]	+ [b]	+ [d]	−
Organic still bottoms	EtOH	0.1–8	−	−	−	−
Dewatered municipal sludge	EtOH	1–16	+ [d]	−	+ [d]	−
Oil-refining waste	NEAT	0.5–4	+ [d]	T	−	−

[a] Extract: NEAT, unadulterated; EtOH, ethanol; DMSO, dimethyl sulfoxide.
[b] 2-fold or greater increase in revertant counts (for the total plate) over spontaneous background values.
[c] Clustering of colonies; distinct localization.
[d] Clustering of colonies; localization detected by grid analysis.
incl, inconclusive findings.
T, toxic.

these findings have been presented elsewhere (Houk and Claxton, 1984; Andon et al., in press).

Results from the mutagenicity testing of the hazardous waste samples are given in Table 2. Test results were accepted as positive if: (1) there occurred a reproducible 2-fold or greater increase in revertant colonies (for the total plate) over spontaneous His^+ revertants, and/or (2) there occurred a reproducible clustering of colonies on the plate. Clustering is an increase in revertant frequency in a specific area of the plate and can occur in one of two ways. Nonrandom clustering appears at localized spots on the plate and is readily detected by the naked eye. Random clustering, on the other hand, is less site-specific and can be detected only with the aid of a grid, followed by a numerical comparison of area revertant counts.

Organic sludge

A relatively nonpolar mutagenic component (which fluoresced blue-white under ultraviolet light) migrated to a high R_f value following chromatography of the organic sludge sample. Fig. 1a illustrates the observed mutagenic response when the chromatogram was tested with TA98 in the absence of metabolic activation.

The diluted counterpart of this sludge, spiked with 11 organic compounds, failed to demonstrate similar activity (Table 2). Two explanations are plausible. The organic compounds which were added to the original sludge could have interfered with the mutagenic expression of the resulting sample. On the other hand, diluting the parent sludge may have masked the original activity, permitting mutagenic expression of the added compounds.

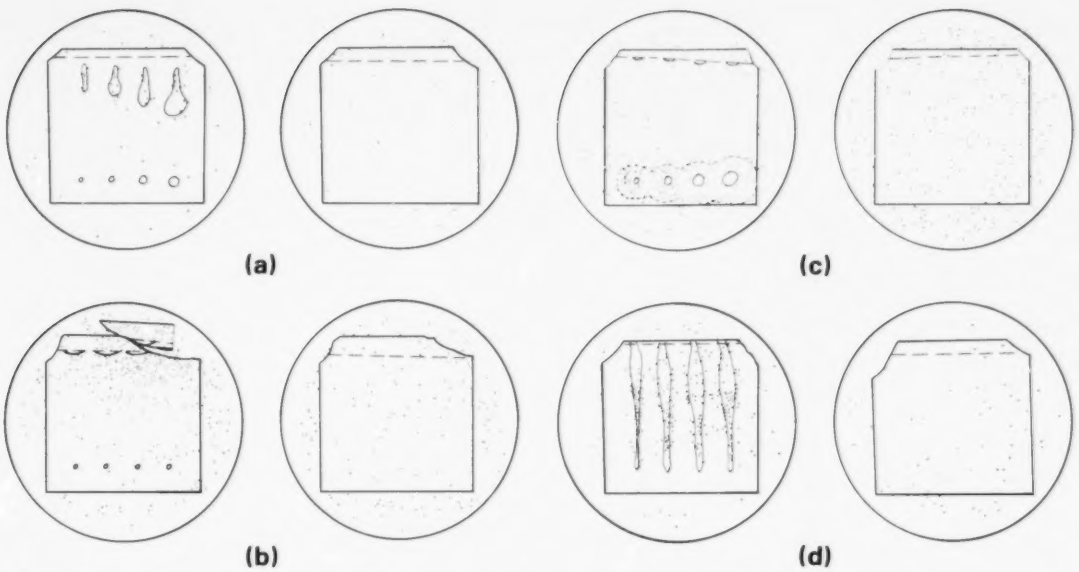

Fig. 1. Separation of mutagenic compounds by the TLC/Salmonella assay. Samples were spotted at increasing volumes across the plate, chromatographed with chloroform, and tested for mutagenic activity. Sample application points and chromatographed spots are indicated (see text for discussion). Negative control plates (spontaneous revertants) are also illustrated. ○, application point; — — —, solvent front. (a) Organic sludge (0.25, 0.5, 1.0, 2.0 μl) tested with TA98, −S9. (b) EDC spent caustic (2, 4, 6, 8 μl) tested with TA100, −S9. Plate was broken during excision of top corners. (c) Herbicide manufacturing acid (0.5, 1.0, 2.0, 4.0 μl) tested with TA100, +S9. (d) Coal gasification tar (unknown volumes) tested with TA98, +S9.

Results from the diluted sludge sample clearly illustrate a potential disadvantage of this assay. Because applied sample volumes must remain small for chromatographic purposes, dilute samples that contain mutagens in low concentrations may go undetected.

EDC spent caustic

The EDC spent caustic contained a mutagen(s) that induced a dose-dependent cluster of revertant colonies in both strains without metabolic activation. Activity was localized around fluorescent orange bands that migrated with the solvent front (Fig. 1b). The migration of this direct-acting mutagen suggests that the compound is relatively nonpolar.

Substantiating evidence for this conclusion has been obtained by an independent analysis of this caustic waste. A methylene chloride extract of this sample tested by the plate incorporation assay demonstrated mutagenic activity in strains TA98 and TA100 without activation (S. Warren, personal communication).

Herbicide manufacturing acid

With activation, the herbicide manufacturing acid produced intense clustering of revertant colonies around the points of sample application (both strains). Clustering was localized within circular fluorescent zones (Fig. 1c). At doses greater than 1 μl, a zone of toxicity surrounded by a ring of revertant colonies was evident. These results indicate that indirect-acting mutagen(s) are present that are toxic to Salmonella when administered at sample doses greater than 1 μl. Results from this sample illustrate a potential advantage to testing small sample volumes; assay interference due to toxicity has been minimized.

The mutagenic constituent present in this herbicide waste was not desorbed by chloroform and consequently did not ascend the TLC plate. Thus, two nonpolar mobile phases described for

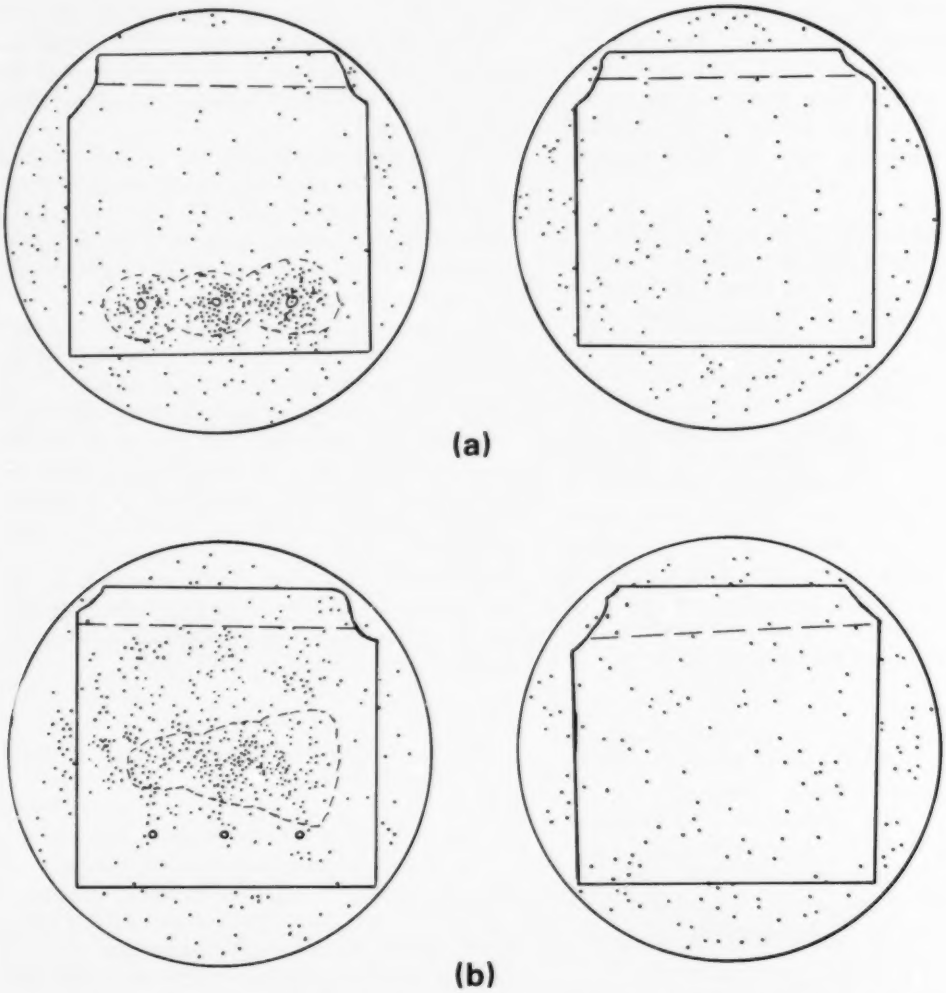

Fig. 2. TLC/Salmonella analysis of the herbicide manufacturing acid chromatographed with different solvent systems. Fluorescent zones (denoted by a dashed line) are located around sample application points in (a) and midway up the plate in (b). Negative control plates (spontaneous revertants) are also illustrated. (a) Sample Volumes: 0.1, 0.25, 0.5 μl; mobile phase: chloroform; mutagenicity testing: TA100 with activation. (b) Sample volumes: 0.1, 0.25, 0.5 μl; mobile phase: DCM/EtOH/water, 10:20:1; mutagenicity testing: TA100 with activation. ○, application point; — — —, solvent front.

pesticide separations were selected to try to enhance migration. The first was a solvent system for separation of chlorinated pesticides (heptane–acetone, 98:2) described by Stephens and Chan (1980). The second was a system that has proven useful in the separation of organophosphorus pesticides (hexane–acetone, 5:1) (Stephens and deVera, 1979). Both solvent systems failed to partition the herbicide waste A polar mobile phase (dichloromethane–ethanol–water, 10:20:1) described by Tomingas et al. (1977) subsequently proved successful in desorbing the mutagenic compound(s). Most revertant activity remained localized midway up the plate within the fluorescent zone (Fig. 2).

The initial localization of revertant activity

around the points of sample application suggests that the mutagen(s) is very polar. Changes in the mobile phase system from nonpolar to polar have supported this assumption. These findings demonstrate the usefulness of testing different solvent systems to more clearly define chemical properties.

Coke plant waste

The neat coke plant waste produced approximately a 2-fold increase in the number of spontaneous His^+ revertants when tested with TA98 in the absence of exogenous activation. Ethanol and DMSO extracts of this sample produced clusters of colonies at points of sample application (figures not included). Independent analysis using the standard plate-incorporation assay supports our findings of mutagenic activity in the extracts (Andon et al., in press). Additional verification of the mutagenic potential of this coke plant waste comes from GC/MS analysis, which revealed the presence of the mutagens/carcinogens benzo[*a*]pyrene (1.12 μg/g), benzo[*b*]- and benzo[*k*]-fluoranthene (0.69 μg/g), and chrysene and benzo[*a*]anthracene (0.5 μg/g) (Miller et al., 1981).

Ink pigment sludge

No mutagenic activity was evident when ink pigment sludge was tested with TA98. With TA100, results proved inconclusive. Although total revertant counts were greater than spontaneous counts (both with and without activation), response was not twice background. Three independent experiments were performed with increasing dosages, but convincing results could not be obtained.

Latex paint waste

Results from the TLC/Salmonella assay of the latex paint waste were negative. However, elemental analysis of this waste indicated the presence of several carcinogenic and mutagenic metals, including cadmium, arsenic and selenium (Miller et al., 1981). In general, the Salmonella reversion assay does not detect mutagenic metals without bioassay modifications, so it follows that the TLC/Salmonella assay would also prove unsuitable for the routine screening of metal-containing wastes.

Coal gasification tar

The coal gasification tar was too viscous to be drawn into a pipette tip. Instead, the tip was touched onto the surface of the sticky substance and a small amount of tar was transferred to the TLC plate. Consequently, applied sample volumes are unknown.

When chromatographed, this sample streaked the length of the plate with no definition of component parts. Under ultraviolet light the streaks fluoresced a deep orange and brown at the application points, yellow-orange midway up the plate, and blue-white at the solvent front.

Mutagenicity testing suggests that this complex sample contains indirect-acting frameshift mutagens that are not particularly water-soluble. This conclusion is based upon the fact that TA98 (+S9) revertants are localized along the length of the visualized streak and are not profuse across the plate (Fig. 1d). There also appear to be direct-acting frameshift mutagens that are more water-soluble, as evidenced by the diffuse response observed on plates without metabolic activation (figure not shown). Two-dimensional chromatography of this sample is recommended to improve the resolution of mutagenic constituents.

Organic still bottoms, EtOH extract

There was no evidence of mutagenic or toxic activity in either strain.

Dewatered municipal sludge, EtOH extract

Revertant clustering was observed in the area of the origin when the chromatogram of the municipal sludge was tested with metabolic activation (both strains). These results suggest the presence of indirect-acting mutagens that are polar and probably water-soluble.

Oil-refining waste

In independent duplicate experiments there appeared a slight dose–response clustering around the origin in TA98 with activation. This effect was evident only at low doses (from 0.5 to 2 μl) and disappeared at volumes of 4 μl and greater. The explanation for this response may be linked to the influence of a fluorescent spot that became larger as sample volumes increased. This separated component appeared to be toxic to the bacteria. No revertant colonies were observed within the confines of the spot, and on one plate small pinpoint

colonies suggestive of toxicity were evident within the area of the spot. An overall reduction of colony counts vis-à-vis control plates was also noted.

Supportive evidence of this sample's toxic potential comes from a study using the CHO cytotoxicity test (Andon et al., in press). This sample produced a greater than 50% reduction both in the cellular ATP concentration and in the viability index in Chinese hamster ovary cells. Because oil refining wastes have been shown to contain significant levels of lead and chromium (U.S. Environmental Protection Agency, 1980), the observed cytotoxic response may ostensibly be linked to these toxic metals.

Analysis of model compounds

Results from the testing of the Salmonella mutagens are given in Table 3. Three patterns of positive response were observed: (1) a profusion of revertant colonies across the plate (as would be expected with highly water-soluble compounds); (2) localized clusters of revertant colonies at points of sample application, responding in a dose-dependent manner; and (3) a dose-dependent ring of revertant colonies around a central toxic zone.

11 of the 14 test compounds were positive, including alkylating agents [methyl methanesulfonate (MMS), cyclophosphamide, β-propiolactone], aromatic amines [2-acetylaminofluorine (2-AAF), 2-anthramine (2-AN)], antitumor agents (daunomycin, streptozotocin), a nitrosamine [*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG)], and a nitroaromatic [2-nitrofluorene (2-NF)]. Two compounds (2-AN and sodium azide) had been previously tested by Bjørseth et al. (1982). Observed patterns of response were similar to those reported by Bjørseth: sodium azide (polar and

TABLE 3

RESULTS FROM TLC/SALMONELLA ANALYSIS OF MODEL COMPOUNDS

Compound	Responsive dose range [a] (μg/application)	Mutagenic activity		
		Result	Strain	Activation
Sodium azide	2–16	+ [b]	TA100	– S9
MMS	2–16	+ [b]	TA100	– S9
β-Propiolactone	100–400	+ [b]	TA100	– S9
2-Nitrofluorene	2–16	+ [c]	TA98	– S9
Cyclophosphamide	2–16	weak + [c]	TA100	+ S9
	20–80	+ [c]	TA100	+ S9
2-AAF	2–16	+ [c]	TA98	+ S9
	20–80	+ [c]	TA100	+ S9
Daunomycin	0.125–1	+ [c]	TA98	– S9
	2–16	toxic	TA98	– S9
Streptozotocin	2–16	+ [d]	TA100	– S9
4-NQO	0.5–16	+ [d]	TA98	– S9
	0.5–16	+ [d]	TA100	– S9
MNNG	2–16	+ [d]	TA100	– S9
2-Anthramine	2–16	+ [d]	TA98	+ S9
	2–16	+ [d]	TA100	+ S9
Benzo[*a*]pyrene	2–80	–	TA98	+ S9
	2–80	–	TA100	+ S9
Epichlorohydrin	2–200	–	TA100	– S9
Dimethylcarbamyl chloride	2–2000	–	TA100	– S9

[a] Responsive dose range does not necessarily reflect the lowest possible or highest achievable dose. It merely indicates those doses tested.

[b] Profusion of revertant colonies across the plate.

[c] Localized clusters of revertant colonies at points of sample application.

[d] Dose-dependent ring of revertant colonies around a central toxic zone.

water-soluble) produced a profusion of revertant colonies across the plate; 2-AN (of intermediate polarity) elicited a dose –response mutagenic effect in which revertant colonies were clustered around points of sample application.

Three compounds — benzo[*a*]pyrene (BaP), epichlorohydrin and dimethylcarbamyl chloride — registered negative responses, even at very high dose levels (Table 3). Previous TLC/Salmonella research on BaP also resulted in negative findings (Bjørseth et al., 1982). As reported by Alfheim et al. (1983) and Møller et al. (1983), this assay system appears unsuitable for the detection of non-polar organics such as unsubstituted PAHs.

The lack of demonstrable mutagenic activity shown by epichlorohydrin and dimethylcarbamyl chloride is presumably linked to their high volatility. These unstable compounds probably volatilized from the chromatography plate prior to testing, thereby precluding detection by the mutagenesis assay.

A nonmutagen (1-naphthylamine, 2–80 μg) was also tested and did not register a mutagenic response.

Conclusion

Our findings demonstrate that the TLC/Salmonella assay described in this paper can detect mutagens in complex hazardous wastes. Of 10 wastes tested, 7 were found to contain mutagenically active compounds. The assay proved to be applicable to a broad range of chemically and physically dissimilar waste types, including acids, caustics, tars, emulsions, sludges, solvents, organics and inorganics. Liquid or near-liquid samples could be applied to the TLC plates without preparatory workup. Solid wastes, however, had to be converted to a form suitable for TLC application and development. The system proved sensitive to microliter volumes of crude test sample.

Additionally, our investigation has shown that detection capabilities cover a wide range of mutagen classes and polarities. Positive responses were elicited from alkylating agents, aromatic amines, antitumor agents, a nitroaromatic and a nitrosamine. Mutagenic activity was detectable in the microgram range. Compounds refractory to analysis by the TLC/Salmonella assay included two mutagens that were volatile and an unsubstituted PAH, a class that does not ordinarily evoke a mutagenic response in a spot test.

Chemical and physical properties of detected mutagens may be inferred from parameters such as localization on the TLC plate (expressed as an R_f value), polarity of the mobile phase, and response to visualization techniques (fluorescence, color, etc.). Fluorescent mutagens were present in the organic sludge, the EDC spent caustic, the herbicide manufacturing acid, and the coal gasification tar. Polar mutagens (those with low R_f values) were detected in the herbicide manufacturing acid, the municipal sludge, and the oil-refining waste, while nonpolar mutagens were detected in the organic sludge and the EDC spent caustic.

Because the TLC/Salmonella assay is a recent approach to analysis of complex mixtures, potential problems have not been thoroughly addressed. Possible sources of error which came to our attention will be discussed here. For example, compounds labile under the conditions of chromatography (volatiles and other chemically unstable compounds) will be lost. Additionally, nondiffusible agents (such as nonpolar organics) and compounds that irreversibly adsorb to the TLC sorbent will fail to yield reliable results. Of related concern is the similarity between the TLC/Salmonella assay and a spot test. Because mutagen detection in the TLC/Salmonella assay mimics that of a conventional spot test, compounds that cannot be detected in spot tests — such as BaP (Ames et al., 1975) — may appear negative when tested by this technique. In addition, the assay may be less sensitive than a standard plate-incorporation assay because relatively few bacteria are exposed to a compound at any particular dose level. Indeed, small total numbers of revertants (relative to a standard plate assay) were observed during our analyses. We attempted to discount this source of bias by conducting replicate testing, but an increase in tester-strain volume may also serve to improve assay sensitivity and reliability.

The TLC/Salmonella assay does offer several important advantages over other coupled fractionation/bioassay schemes. The procedure is very rapid. Fractionation of the samples by chromatography can be completed within 10–20 min. Mutagenicity results are documented after 72 h. Sam-

ples can be applied to the system in their neat state; thus, extraction procedures are obviated except for samples that cannot be chromatographed in their crude form (e.g., solids). Compounds that may exert synergistic or antagonistic effects are separated from the complex sample during chromatography and are detected independently. Problems with sample toxicity are minimized. [When extracts of the coal gasification tar included in this study were tested by the standard plate-incorporation assay, toxicity precluded detection of mutagenic compounds at the doses tested (S. Warren, personal communication). Our results indicated a mutagenic response in both strains.]

In summary, the TLC/Salmonella assay represents a simple screening tool for the qualitative detection of mutagens in complex environmental samples. The speed and economy with which results can be obtained make it useful for routine testing and for processing large numbers of samples. The presumptive identification of mutagenic constituents in a sample can lead to a systematic selection of that sample for more conclusive chemical or biological evaluation.

Acknowledgements

Special gratitude is expressed to Dr. Alvis G. Turner of the Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, for his participation in this project.

This paper does not necessarily reflect the views of the Environmental Protection Agency, and no official endorsement should be inferred.

References

Alfheim, I., J.G.T. Bergstrom, D. Janssen and M. Møller (1983) Mutagenicity in emissions from coal- and oil-fired boilers, Environ. Health Perspect., 47, 177–187.

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutation Res., 31, 347–364.

Andon, B.M., M. Jackson, V. Houk and L. Claxton (in press) The evaluation of chemical and biological methods for the identification of mutagenic and cytotoxic hazardous waste samples, in: J.K. Petros Jr., W.J. Lacy and R.A. Conway (Eds.), Hazardous and Industrial Solid Waste Testing: Fourth Symposium, American Society for Testing and Materials STP 886, Philadelphia, 1985.

Battelle Columbus Laboratories (1981) Final Report on the Collaborative Study for the Evaluation of a Selected Method for Hazardous Waste Analysis, 8 Dec. 1981.

Bjørseth, A., G.E. Carlberg and M. Møller (1979) Determination of halogenated organic compounds and mutagenicity testing of spent bleach liquors, Sci. Total Environ., 11, 197–211.

Bjørseth, A., G. Eidså, J. Gether, L. Landmark and M. Møller (1982) Detection of mutagens in complex samples by the Salmonella assay applied directly on thin layer chromatography plates, Science, 215, 87–89.

Claxton, L.D., M. Kohan, A. Austin and C. Evans (1981) The Genetic Bioassay Branch protocol for bacterial mutagenesis including safety and quality assurance procedures, Health Effects Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC.

Clevenger, T.E., D. D. Hemphill, K. Roberts and W.A. Mullins (1983) Chemical composition and possible mutagenicity of municipal sludges, J. Water Pollut. Control Fed., 55, 1470–1475.

Commoner, B., P. Mdayastha, A. Bronsdon and A.J. Vithayathil (1978) Environmental mutagens in urban air particulates, J. Toxicol. Environ. Health, 4, 59–77.

Dehnen, W., N. Pitts and R. Tomingas (1977) The mutagenicity of airborne particulate pollutants, Cancer Lett., 4, 5–12.

Douglas, G.R., E.R. Nestmann, J.L. Betts et al. (1980) Mutagenic activity in pulp mill effluents, in: R.L. Jolley, W.A. Brungs and R.B. Cummings (Eds.) Water Chlorination: Environmental Impact and Health Effects, Vol. 3, Ann Arbor Science Publications, Ann Arbor, MI, pp. 865–881.

Epler, J.L., F.W. Larimer, C.E. Nix, T. Ho and T.K. Rao (1977) Comparative mutagenesis of test materials from the synthetic fuel technologies, in: D. Scott, B.A. Bridges and F.H. Sobels (Eds.) Progress in Genetic Toxicology, Elsevier, Amsterdam, pp. 275–282.

Epler, J.L., F.W. Larimer, T.K. Rao, C.E. Nix and T. Ho (1978a) Energy-related pollutants in the environment: Use of short-term tests for mutagenicity in the isolation and identification of biohazards, Environ. Health Persp., 27, 11–20.

Epler, J.L., J.A. Young, A.A. Hardigree, T.K. Rao, M.R. Guerin, I.B. Rubin, C.-H. Ho and B.R. Clark (1978b) Analytical and biological analyses of test materials from the synthetic fuel technologies, I. Mutagenicity of crude oils determined by the *Salmonella typhimurium*/microsomal activation system, Mutation Res., 57, 265–276.

Hopke, P.K., M.J. Plewa, J.B. Johnston, D. Weaver, S.G. Wood, R.A. Larson and T. Hinesly (1982) Multitechnique screening of Chicago municipal sewage sludge for mutagenic activity, Environ. Sci. Technol., 16, 140–147.

Houk, V.S. (1984) Screening complex hazardous wastes for mutagenic activity using the TLC/Ames assay, Master's Thesis, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC.

Houk, V.S., and L.D. Claxton (1984) Application of the Salmonella/TLC plate assay for screening complex hazardous waste streams, presented at the 15th Annual Meeting of the Environmental Mutagen Society, 19–23 February, Montreal, Canada.

Kamra, O.P., E.R. Nestmann, G.R. Douglas, D.J. Kowbel and T.R. Harrington (1983) Genotoxic activity of pulp mill effluent in Salmonella and *Saccharomyces cerevisiae* assays, Mutation Res., 118, 269–276.

Maugh, T.H. (1979) Toxic waste disposal a growing problem, Science, 204, 819–823.

Miller, H.C., K.H. James, W.K. Dickson, M.D. Neptune and M.H. Carter (1981) On evaluation of methodology for the survey analysis of solid wastes, in: R.A. Conway and B.C. Malloy (Eds.), Hazardous Solid Waste Testing: First Conference, American Society for Testing and Materials, Philadelphia, pp. 240–266.

Møller, M., I. Alfheim, G. Lofroth and E. Agurell (1983) Mutagenicity of extracts from typewriter ribbons and related items, Mutation Res., 119, 239–249.

Nestmann, E.R., E.G.H. Lee, T.I. Matula, G.R. Douglas and J.C. Mueller (1980) Mutagenicity of constituents identified in pulp and paper mill effluents using the Salmonella/mammalian-microsome assay, Mutation Res., 79, 203–212.

Pederson, T.C., and Siak, J.-S. (1980) Characterization of direct-acting mutagens in diesel exhaust particulates by thin layer chromatography, Research Publication, Biomedical Science Department, General Motors Research Labs, Warren, MI, 16 April 1980.

Pelroy, R.A., and Wilson, B.W. (1981) Relative concentrations of polyaromatic primary amines and azaarenes in mutagenically active nitrogen fractions from a coal liquid, Mutation Res., 90, 321–335.

Pitts, J.N., K.A. Van Cauwenberghe, D. Grosjean, J.P. Schmid, D.R. Fitz, W.L. Belser, G.B. Knudson and P.M. Hynds (1978) Atmospheric reactions of polycyclic aromatic hydrocarbons: facile formation of mutagenic nitro derivatives, Science, 202, 515–519.

Rannug, U., D. Jenssen, C. Ramel, K.E. Eriksson and K. Kringstad (1981) Mutagenic effects of effluents from chlorine bleaching of pulp, J. Toxicol. Environ. Health, 7, 33–47.

Rao, T.K., J. Young, A. Hardigree, W. Winton and J. Epler (1978) Analytical and biological analyses of test materials from the synthetic fuel technologies, II. Extended genetic and biochemical studies with mutagenic fractions. Mutation Res., 58, 185–191.

Rappóport, S., M. Richard, M. Hollstein and R. Talcott (1979) Mutagenic activity in organic wastewater concentrates, Environ. Sci. Technol., 13, 957–961.

Somani, S.M., R.G. Teece and D.J. Schaeffer (1980) Identification of cocarcinogens and promoters in industrial discharges into and in the Illinois River, J. Toxicol. Environ. Health, 6, 315–331.

Sparacino, C.M. (1983) Preparation and fractionation/characterization of hazardous waste samples for toxicological studies, Draft Final Report, Analytical and Chemical Sciences Division, Research Triangle Institute, Research Triangle Park, NC.

Stephens, R.D., and J.J. Chan (1980) The characterization of hazardous wastes by thin layer chromatography, in: J.C. Touchstone and D. Rogers (Eds.) Thin Layer Chromatography: Quantitative Environmental and Clinical Applications, Wiley, New York, pp. 363–369.

Stephens, R.D., and E.R. deVera (1979) Analysis of hazardous wastes, U.S. Environmental Protection Agency, Municipal Environmental Research Lab., Cincinnati, OH.

Stinnett, S., D. Noble, E. Brown and H. Love (1981) Mutagenicity testing of industrial wastes from representative organic chemical industries, NTIS, PB81-155574, Springfield, VA.

Tabor, M.W., and J.S. Loper (1980) Separation of mutagens from drinking water using coupled bioassay/analytical fractionation, Int. J. Environ. Anal. Chem., 8, 197–215.

Tomingas, R., G. Voltmer and R. Bednarik (1977) Direct fluorometric analysis of aromatic polycyclic hydrocarbons on thin layer chromatograms, Sci. Total Environ., 7, 261–267.

U.S. Department of Commerce (1983) National Bureau of Standards, Report of Analysis on an organic quality assurance sludge material, 21 January 1983, Washington, DC.

U.S. Environmental Protection Agency (1980) Resource Conservation and Recovery Act, Subtitle C, Background Document, "Petroleum Refining", U.S. Government Printing Office, Washington, DC, 19 May 1980.

Vianna, N.J., and A.K. Polan (1984) Incidence of low birth weight among Love Canal residents, Science, 226, 1217–1219.

Warner, J.F., B.J. Hidy, G.A. Jungclaus, M.M. McKown, M.P. Miller and R.M. Riggin (1981) Development of a method for determining the leachability of organic compounds from solid wastes, in: R.A. Conway and B.C. Malloy (Eds.) Hazardous Solid Waste Testing: First Conference, American Society for Testing and Materials, Philadelphia, pp. 40–60.

Warren, C.S. (1981) Hazardous Waste: An Overview, in: J.P. Collins and W.D. Saukin (Eds.) The Hazardous Waste Dilemma: Issues and Solutions, American Society of Civil Engineers, New York, pp. 5–15.

Wilson, B.W., R. Pelroy and J.T. Cresto (1980) Identification of primary aromatic amines in mutagenically active subfractions from coal liquefaction materials, Mutation Res., 79, 193–202.

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

This article documents research that was conducted under the auspices of the U.S. Environmental Protection Agency (USEPA). As an arm of the government, the USEPA has a paid up, non-exclusive, irrevocable, worldwide license to reproduce, prepare derivative works, and distribute to the public all of the material contained herein, and to grant others acting on its behalf the right to do so. The USEPA grants to the National Technical Information Service, Department of Commerce, the right to act as its agent in the distribution of this material.